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Low prevalence of metallo-beta-lactamase in *Pseudomonas aeruginosa* isolated from a tertiary burn care center in Tehran

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Abstract

Production of metallo-beta-lactamase (MBL) is one of the main mechanisms for resistance in carbapenem antibiotics. Detection of MBL-producer *Pseudomonas aeruginosa* is crucial in preventing its spread to other gram-negative bacteria. The aim of this study was to evaluate combination disc (CD) for identification of MBL-producer *P. aeruginosa* by polymerase chain reaction (PCR). A total of 255 imipenem resistant *P. aeruginosa* were collected from burn patients. Antibiotic susceptibility testing was conducted after purification and identification. Double-disc synergy test (DDST) with EDTA and combination disc test (CDT) with dipicolinic acid were performed for phenotypic detection of MBL and the PCR was carried out for bla_{VIM}, bla_{IMP}, $bla_{NDM\text{-}1}$, $bla_{SPM\text{-}1}$ genes. DDST with EDTA was negative in all cases, but 161 isolates were positive in CDT with dipicolinic acid. Further, bla_{VIM} and bla_{IMP} were detected in five and four strains, respectively. None of the isolates were positive for $Bla_{NDM\text{-}1}$ and $bla_{SPM\text{-}1}$. The results of this study showed that the prevalence of MBL is low in imipenem resistance *P. aeruginosa* and that other mechanisms could be involved in resistance to imipenem in this bacterium.

Keywords

metallo-beta-lactamases, phenotypic detection, *Pseudomonas aeruginosa*

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Introduction

Pseudomonas aeruginosa is an obligate aerobic, gram-negative opportunist pathogen, which is able to grow and survive in hospital environment. This bacterium also plays an important role in a lot of severe infections, especially in immunocompromised and burn patients admitted to the burns ward and contains acquired and intrinsic antibiotic resistant genes.[1–3] *P. aeruginosa* is the second leading cause of nosocomial infections in hospitalized burn patients. *P. aeruginosa* is a highly adaptable micro-organism that can rapidly develop resistance to different types of broad-spectrum antibiotics. It can grow in hospital environments characterized by heavy antimicrobial use, and consequently it can be transmitted rapidly among hospitalized burn patients.[1–3]

Carbapenems are often used as a final choice for antibiotic therapy in treating infectious diseases associated with extended spectrum beta-lactamase (ESBL) producing gram-negative bacteria such

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P. aeruginosa and *Acinetobacter baumannii*.[4,5] The carbapenemases have been organized based on amino acid homology in the Ambler molecular classification system. Class A, C, and D beta-lactamases all share a serine residue in the active site, while Class B enzymes require the presence of zinc for activity (and hence are referred to as metallo-beta-lactamases). Classes A, B, and D are of greatest clinical importance among nosocomial pathogens. The main mechanism of resistance in carbapenems is the production of carbapenemase by gram-negative bacteria.[4,6] Metallo-beta-lactamase (MBL) is a class B beta-lactamase, which can hydrolyze carbapenem and other beta-lactams apart from Monobactam.[4,6,7]

The first isolate of MBL producer *P. aeruginosa* was reported in Japan in 1991 and since it has been reported worldwide.[8] The most common MBLs are IMP-type carbapenemase (IMP) and Verona integron-encoded metallo-β-lactamase (VIM) types.[9] The bla_{VIM} and bla_{IMP} genes can spread from one *Pseudomonas* to another as well as *Enterobacteriacea*, since they are inserted into a plasmid-born integron class 1 or 3.[9] Various phenotypic methods for detection of MBL-producer isolates have been suggested such as using chelating agents (i.e. Ethylenediaminetetraacetic acid [EDTA]) and some MBL inhibitor like dipicokinic acid,[10–16]), nevertheless the specificity and sensitivity of these methods can be variable.[11]

DDST with imipenem-EDTA and CDT using imipenem and dipicolinic acid as MBL inhibitors are two of the most common methods utilized in various studies.[10–12] The aim of this study was molecular and phonotypical detection of MBL in *P. aeruginosa* strains isolated from burn patients.

Materials and methods

Bacterial specimen

In this study, 255 *P. aeruginosa* were isolated from burn patients (with at least 1 week of hospitalization) in Motahari Hospital (a referral burns center), Tehran, Iran from June to December 2013. Bacteria were collected from wound infections of hospitalized burn patients. Specimens were collected from male and female wards in hospital. The samples were collected by swabbing burn wound exudates, and immediately transported in transport culture media under standard conditions to the central laboratory of the Antimicrobial Resistance Research Center. *P. aeruginosa* ATTCC27853 was used as the negative control. Isolated bacteria species were identified by specific biochemical and microbiological tests such as oxidase, TSI, and glatinase. Polymerase chain reaction (PCR) was used as a confirmatory test for identification of *P. aeruginosa* strains using specific primers for *oprI* for genus and *oprL* for the species. *P. aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 19606 were used as positive and negative control, respectively.

Antibiotic susceptibility testing

The antibiotic susceptibility testing was performed by disc diffusion method on Mueller–Hinton agar using cefotaxime (30 μg), ceftazidime (30 μg), aztreonam (30 μg), imipenem (10 μg), ticarcillin (75 μg), ticarcillin-clavulanic acid (75/10 μg), piperacillin (100 μg), piperacillin-Tazobactam (100/10 μg), ciprofloxacin (5 μg), gentamicin (10 μg), tobramycin (10 μg), kanamycin (30 μg), amikacin (30 μg), colistin (10 μg), tetracycline (30 μg), and trimethoprim (5 μg) according to clinical and laboratory standards institute (CLSI) 2011. Standard antibiotics discs used in this study were purchased from MAST Company (Mast Diagnostics, UK).

Phenotypic detection of metallo-beta-lactamase

Double-disc synergy test. At first, 0.5 M of EDTA was prepared by dissolving 186.1 g in 1 L of distilled water and pH 8 adjusted by NaOH. Then EDTA 750 μg/disc was prepared. Also, the inhibition zone of that was measured alone. In the next step, the DDST was conducted by imipenem (10 μg) and EDTA distinctly, which was placed with a distance of 20 mm center-to-center with imipenem (10 μg). The strain with increasing size in the imipenem (10 μg) inhibition zone towards EDTA is considered a MBL producer in DDST.[11–14]

Combination disc test

In the CDT, imipenem alone and imipenem (10 μg) impregnated with dipicolinic acid (1000 μg/disc) were examined. The strains with an increase of $\geqslant$5 mm in the inhibition zone around the imipenem (10 μg) plus dipicolinic acid against imipenem (10 μg) alone were considered a MBL producer.[10,16]

PCR for detection of MBL genes

All strains were examined for detection of bla_{VIM}, bla_{IMP}. New Delhi metallo-beta-lactamase-1 (bal_{NDM-1}) and Sao Paulo metallo-beta-lactamase-1

Table 1. Primer sequences and their amplicon sizes for metallo-beta-lactamase genes.

Genes	Sequence (5'→3')	Amplicon size (pb)	Reference
oprI-F1	ATGAACAACGTTCTGAAATTCTCTGCT	249	14
oprI-R1	CTTGCGGCTGGCTTTTTCCAG		
oprL-F1	ATGGAAATGCTGAAATTCGGC	504	
oprL-R1	CTTCTTCAGCTCGACGCGACG		
bla_{VIM}–*F*	ATGTTAAAAGTTATTAGTAGT	801	15
bla_{VIM} -*R*	CTACTCGGCGACTGAGCGAT		
Bla_{IMP}-*F*	GATGGTGTTTGGTCGCATA	139	16
Bla_{IMP}-R	CGAATGCGCAGCACCAG		
bla_{NDM-1}-*F*	CCCGGCCACACCAGTGACA	129	
bla_{NDM-1}-R	GTAGTGCTCAGTGTCGGCAT		17
bla_{SPM-1}-*F*	GGGTGGCTAAGACTATGAAGCC	447	
bla_{SPM-1}-*R*	GCCGCCGAGCTGAATCGG		

Table 2. Antibiotic resistance patterns of *P. aeruginosa* isolates.

Patterns	Antibiotic resistance patterns	n (%)
1	CTX CAZ AT CEF IMI PTZ PYR TC TC-C GM AK TO K TM CI	112 (49.9)
2	CTX CAZ AT CEF IMI PTZ PYR TC TC-C GM AK TO K TM CI T	92 (41)
3	CTX CAZ AT CEF IMI PYR TC TC-C GM AK TO K TM T	3 (1.3)
4	CTX AT CEF IMI PTZ PYR TC-C AK K TM CI T	3 (1.3)
5	CTX IMI PTZ PYR TC TC-C GM AK TO K TM CI	3 (1.3)
6	CTX IMI PYR TC TC-C AK TO K TM CI	3 (1.3)
7	CTX CAZ CEF IMI TC TC-C GM AK TO K TM CI	3 (1.3)
8	CTX CAZ AT CEF IMI TC TC-C GM AK TO K TM CI	3 (1.3)
9	CTX AT CEF IMI PTZ PYR TC TC-C GM AK TO K TM CI T	3 (1.3)

(bal_{SPM-1}) by PCR with primers and PCR program were shown in Table 1.

PCR was performed for each sample with the following compounds: 1× concentration Specific PCR buffer, 0.4 mM of dNTPs mix, 0.7 mM of $MgCl_2$, 1.6 M of each primer, one unit of Taq polymerase enzyme, 2 µL of DNA, and sterile distilled water to get 25 µL as a final volume. Finally, the PCR products were evaluated on a 1.5% agarose gel.

PCR (bla_{VIM}, bla_{IMP} genes) was performed in the following condition. The DNA thermocycle was programmed as follows: the first denaturation at 94°C for 5 and 10 min for VIM and IMP, respectively; 30 cycles of 94°C for 60 and 40 se for VIM and IMP, respectively; annealing at 55°C for 60 and 40 s VIM and IMP, respectively; extension at 72°C for 60 s; and at last the final extension at 72°C for 5 and 7 min for VIM and IMP, respectively.

Internal positive controls were considered in this study. The first positive strain is sequenced and used for positive control in all tested strains.

Multiplex PCR (bal_{NDM-1} *and* bal_{SPM-1} genes) was performed in the following condition. The DNA thermocycle was programmed as follows: the first denaturation at 94°C for 60 s for set; 30 cycles of 94°C for 30 s; annealing at 55°C for 60 s; extension at 72°C for 60 s; and at last the final extension at 72°C for 60 s.

Results

Two hundred and fifty-five strains were confirmed as *P. aeruginosa* by specific biochemical tests and subsequently by PCR. The results were interpreted according to CLSI 2011 AST guidelines. All tested strains were resistant to imipenem. The antibiotic resistant patterns were shown in Table 2.

AK: amikacin; AT, aztreonam; CAZ: ceftazidime; CEF: cefepime; CI: ciprofloxacin; CTX: cefotaxime, GM: gentamicin, IMI : imipenem; K: kanamycin; PRL: piperacillin-Tazobactam; PTZ: piperacillin; T: tetracycline; TC: ticarcillin; TC-c: ticarcillin-clavulanic acid; TM: trimethoprim, TO: tobramycin.

Antibiotic resistance pattern 1 (resistant to all tested antibiotics except colistin) was the most observed patterns in both two studied wards.

The first five antibiotics prescribed that were consided as a first line of treatment were: cefepime, ciprofloxacin, amikacin, meropenem, imipenem, piperacillin-Tazobactam, and gentamicin. In total, 161 (63%) of imipenem-resistant strains were shown increasing the diameter of the inhibition zone by at least 5 mm with imipenem-dipicolinic acid compared with imipenem alone in CDT. Simultaneously, dipicolinic acid alone increased the inhibition zone to 12 mm (Figure 1).

None of them maintained synergistic effects between EDTA alone and imipenem alone. Direct sequencing of PCR amplified products was carried out using ABI 3730X capillary sequencer (Genfanavaran, Macrogen, Seoul, Republic of Korea). Sequence analysis showed that bla_{VIM} and bla_{IMP} genes were detected in five and four tested strains, respectively (Figures 2 and 3).

All of these nine strains which were confirmed by PCR as MBL producers, had positive results in CDT. None of the CDT negative strains had MBL genes, but 152 out of 161 which were CDT positive have not shown the specific band for MBL-tested genes after PCR and gel electrophoresis. $bla_{NDM-1}$and bla_{SPM-1} have been not detected.

Figure 1. The inhibition zone of dipicolinic acid disc alone.

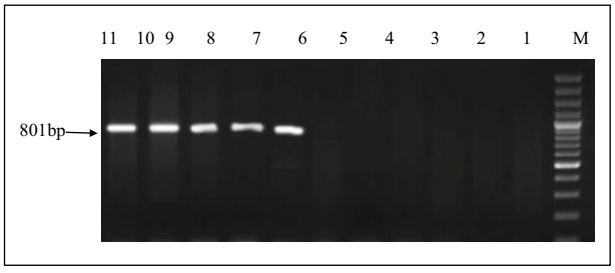

Figure 2. PCR amplification fragments for detection of *vim* gene among *P. aeroginosa* isolates. Lanes 1–5: negative *VIM1*; lane 6: Negative control for *VIM1* gene; lanes 7–10: positive *VIM1* strains; lane 11: positive control; M: 1 kb marker.

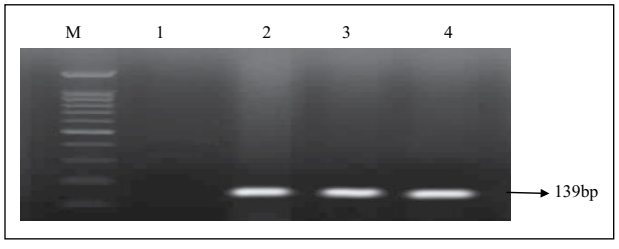

Figure 3. PCR amplification fragments for detection of *imp* gene among *P. aeroginosa* isolates Lane 1: negative control; lane 2: positive control for *imp* gene; lanes 3 and 4: positive *imp* strains; M: 1 kb marker.

Discussion

Prevalence of carbapenem resistance *P. aeruginosa* is increasing mainly due to potential MBL production, which ultimately can cause high morbidity and mortality, particularly among hospitalized burn patients.[21] The results of the study in 2014 in Colombia indicated that 60% of carbapenem resistant *P. aeruginosa* was a VIM producer and none of them was positive for IMP and NDM.[22] In this study, as in our results, NDM-producer *P. aeruginosa* has been not detected, but the prevalence of VIM is higher than in our study. On the other hand, IMP has shown less prevalence in comparison to our results. Overall 60% of imipenem resistant *P. aeruginosa* in Colombia has been confirmed as a MBL producer, but 3.5% of imipenem resistant *P. aeruginosa* in the our study were MBL producers. This differentiation of MBL prevalence can relate to a kind of antibiotic therapy in two different countries and may be associated with a specific mechanism which is prevalent in various countries. It can be considered that in 2010 in Iran 23% of non-susceptible imipenem *P. aeruginosa* had bla_{VIM} and bla_{IMP} genes[23] and this prevalence higher compared to our tested isolates in 2013. These different results in two different time frames but in the same country can indicate the change of carbapenem resistant

mechanism in *P. aeruginosa* and could be related to the existence of another resistant mechanism.

DDST methods with imipenem and EDTA and CDT with imipenem and dipicolinic acid are used as two ordinary phenotypic methods for detection of MBLs.[11] In a previous study in Iran, the DDST test was used for the detection of MBL in *Pseudomonas* spp., which showed that 23% of non-susceptible imipenem isolates have bla_{VIM} and bla_{IMP} genes.[23] In 2011 in Norway, the sensitivity and specificity of CDT with dipicolinic acid were 100% in *K. pneumoniae* strains.[10]

In 2013 in Iran, 87.8% of tested imipenem resistant isolates were confirmed to be MBL producer based on DDST. bla_{IMP} and bla_{VIM} were detected in 24.4% and 56% of strains, respectively. This differention in the results of this study and the current study can be associated with the location of sampling in patients. On the other hand, these two studies were conducted in two different hospitals in two different cities with different antibiotic therapy policies.[24]

It can be noted that one antibiotic-resistant pattern was the most observed pattern in the two studied wards and it can indicate the spread of one bacterial clone in the two wards.

In the present study, no synergistic effect was observed using the DDST techniques, but the CD test was positive in 63% of imipenem resistant *P. aeruginosa*. Our findings also showed that dipicolinic acid alone tested for *Pseudomonas* strains had an inhibition zone up to 12 mm. Thus, an increase of 5 mm in the inhibition zone around imipenem with dipicolinic acid against imipenem alone cannot explain the synergistic effect of these two materials. In this regard, the specificity of dipicolinic acid was 81% for the detection of MBL in study of Pasteran et al.[16]

On the other hand, molecular tests for the presence of genes bl_{NDM} and bla_{SPM} were negative and only 3.5% of isolates had bla_{VIM} and bla_{IMP} genes. It can explain the other carbapenem resistant mechanisms among *P. aeruginosa* like the efflux pump[25] and over-expression of AmpC combined with porin loss.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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